***ANNEXURE* – I**A

PROFORMA

Patient's Name :	Date :
Age / Gender :	OPD/ IPD No. :
Occupation :	Contact No. :
Address :	
Refered by Dr. :	Hospital :

Patient's Chief Complaints	**Yes**	**No**
Fever with duration		
Cough with duration		
Cough with expectoration		
Hemoptysis		
Night sweats		
Loss of weight		
Loss of appetite		
Fatigue		
Chest pain		
Breathessness		
Sleep		
Other complains		
History of Patient		
History of contact with active TB patient		
Family history		
Smoking habit		
Alcohol		
Treatment history		
Past history of ATT		

ANNEXURE – IB

CLINICAL EXAMINATION	FINDINGS
General Physical examination 1. Weight 2. Pallor 3. Icterus 4. Lymph nodes 5. Kylonychia 6. Odema	
Systemic examination 1. Respiratory system 2. Central Nervous system 3. CardioVascular system 4. Gastro intestinal system	

INVESTIGATIONS	FINDINGS
Chest X-ray	
Tuberculin skin test	
Direct smear (Z-N stain)	
Conc. Smear (Z-N stain)	
RSC-I culture	
RSC-II culture	
MGIT culture	
GenProbe assay	
DRUG SUSCEPTIBILITY TESTING	
Slide DST- I RIF INH	
Slide DST- II RIF INH	
MGIT DST RIF INH	
MTBDR*plus* *rpo*B genes *inh*A genes *cat*G genes	

ANNEXURE – II

SMEAR PREPARATION

1. A new unscratched glass slide will be selected will be labelled with the laboratory serial number with a diamond marking pencil.
2. A smear will be made from sediment by sterilised wood stick. A good smear is spread evenly, 2cm X 3cm in size and is neither too thick nor too thin.
3. The slide allowed to air dry for 15-20 minutes. The slide will be fixed by passing it over a flame 3-5 times for 3-4 seconds each time.

ANNEXURE – IIIA

NALC – NaOH CONCENTRATION METHOD

MATERIALS REQUIRED

- Disposable 50 ml plastic tubes (Falcon tubes)
- Sodium Hydroxide
- Tri-sodium citrate
- NALC powder
- Potassium di-hydrogen phosphate (KH_2PO_4)
- Di-sodium hydrogen phosphate (Na_2HPO_4) anhydrous

Preparation of NALC-NaOH solution

- Prepare 4% NaOH solution by dissolving 4g NaOH pellets into 100 ml distilled water. Sterilize by autoclaving.
- Prepare 2.9% sodium citrate solution by dissolving 2.9 g sodium citrate in 100 ml distilled water. Sterilize by autoclaving.
- Mixing prior to use, mix equal quantities of NaOH and sodium citrate solution. Prepare only as much volume as can be used in a day.
- Add NALC powder to achieve a final concentration of 0.5% (for 100 ml NaOH-Sodium citrate solution, add 0.5 g NALC powder).
- Mix well and use the same day.

Preparation of Phosphate buffer (pH 6.8, 0.067 M)

- **Solution A:** Dissolve 9.47g of anhydrous disodium phosphate in 1000 ml distilled water.
- **Solution B:** Dissolve 9.07 g monopotassium di-hydrogen phosphate in 1000 ml distilled.
- Mix equal quantities of the two solutions.
- Check the pH. The final pH should be 6.8.
- Adding more solution A will raise the pH; more solution B will lower the pH.
- Sterilize by autoclaving.

ANNEXURE – IIIB

Specimen processing

- If specimen is not collected in a 50 ml centrifuge tube, transfer it to a 50 ml centrifuge tube with a screw cap.
- Add NaOH-NALC-sodium citrate solution in a volume equal to the quantity of specimen. Tighten the cap. Vortex lightly or hand mix for about 15-30 seconds.Invert the tube so the whole tube is exposed to the NaOH-NALC solution.
- Wait 15-20 minutes (up to 25 minutes maximum) after adding the NaOH-NALC solution. Vortex lightly or hand mix/invert every 5-10 minutes or put the tubes on a shaker and shake lightly during the whole time.
- Make sure the specimen is completely liquefied. If still mucoid, add a small quantity of NALC powder (30-35 grams) directly to the specimen tube. Mix well.
- At the end of 15-20 minutes, add phosphate buffer (pH 6.8) up to the top ring on the centrifuge tube (plastic tube has a ring for 50 ml mark). Mix well (lightly vortex or invert several times).
- Centrifuge the specimen at a speed of 3000 g or more for 15-20 minutes. Use of refrigerated centrifugation at a higher speed is known to increase recovery of mycobacteria.
- After centrifugation, allow tubes to sit for 5 minutes to allow aerosols to settle. Then carefully decant the supernatant into a suitable container containing a mycobactericidal disinfectant. Make sure the sediment is not lost during decanting of the supernatant fluid.
- Add a small quantity (1-2 ml) phosphate buffer (pH 6.8) and resuspend the sediment with the help of a pipette or vortex mixer.
- Use the resuspended pellet for making smears and for culture.

ANNEXURE – IVA

ZIEHL-NEELSEN STAINING

MATERIAL REQUIRED:-

1. Strong carbolfuchsin(1%): It is the primary stain and consists of 0.3 gms of basic fuchsin in 10ml of 95% ethanol, 5ml phenol and 95ml of distilled water.
2. Sulphuric acid (25%); -It is the decolorizer.
3. Methylene blue (0.1%): - It is the counter stain and consists of 0.3 gms of methylene blue in 100ml of distilled water.

PREPARATION OF SMEARS AND ZN STAINING TECHNIQUE:-

1. A new unscratched slide was selected and labeled with the Laboratory Serial number with a diamond marking pencil.
2. A smear was prepared from supernatant fluid and sediment also by the sterilized inoculating loop. A good smear was spread evenly, 2 cms x 3 cms in size and was neither too thick nor too thin.
3. The slide was allowed to air dry for 15–30 minutes.
4. The slide has been fixed by passing it over a flame 3–5 times for 3–4 seconds each time.
5. 1% filtered carbolfuchsin was poured to cover the entire slide.
6. It has then been gently heated with carbolfuchsin on it, until vapors rise. Making sure it does not boil.
7. Carbolfuchsin has left on the slide for 5 minutes.
8. The slide was gently rinsed with tap water until all free carbolfuchsin stain was washed away.
9. 25% Sulphuric acid has then been poured onto the slide and has been allowed to stand for 2–4 minutes. The slide was gently rinsed with tap water and tilted to drain off the water.
10. Counterstain 0.1% methylene blue was poured onto the slide and left for 30 seconds. Then the slide was rinsed gently with tap water and it was allowed to dry.
11. The slide was finally examined under 100x lens of light microscope.[12]

ANNEXURE – IVB

GRADING GUIDELINES

Record exact number of AFB seen in 100 fields.

Examination finding	**Result as recorded**	**Grading**	**No. of fields examined**
> 10 AFB/oil immersion field	Positive	3+	20
1–10 AFB/oil immersion field	Positive	2+	50
10–99 AFB/100 oil immersion fields	Positive	1+	100
1–9 AFB/100 oil immersion fields	Positive	Scanty*	100
No AFB/100 oil immersion fields	Negative	Negative	100

ANNEXURE – VA

MGIT CULTURE PROCEDURE [MGIT Procedure Manual]

A. GENERAL SAFETY CONSIDERSTIONS

1. Biosafety Level 3 practice, containment equipment, and facilities are required as recommended at the CDC.
2. Perform all specimen processing and positive tube staining or sub culturing in a Biological Safety Cabinet. Appropriate protective clothing, including glove and masks, should be worn.
3. Dropped tubes should be examined carefully. If damage is seen, the tube should be discarded.
4. Before discarding, place all contaminated BBL MGIT tubes and other materials in a puncture proof container. Autoclave and dispose of according to your facility's procedure.

B. PROCESSING NEW CULTURES

INOCULATION OF MGIT TUBES:

1. Reconstitute a lyophilized vial of BBL MGIT PANTA antibiotic mixture with15 mL of Growth Supplement.
2. Label the MGIT tube with the specimen number.
3. Unscrew the cap and aseptically add 0.8 mL of MGIT Growth Supplement/MGIT PANTA antibiotic mixture.
4. Add 0.5 mL of the digested, decontaminated, and concentrated specimen suspension.
5. Tightly recap the tube and mix well and entered into the instrument will be automatically tested for the duration of the recommended 42-day testing protocol.
6. Subculture the positive tubes on Middlebrook 7H10 agar and prepare an acid fast smear.

ANNEXURE – VB

C. PROCESSING OF POSITIVE MGIT TUBE(s): NOTE: All steps should be performed in a biological safety cabinet.

1. Remove the positive MGIT tube from the instrument and transport to an area using BSL III bio safety cabinate.
2. Using a sterile transfer pipette, remove an aliquot from the bottom of the tube (approx. 0.1 mL) for stain preparations (AFB and Gram stains),
3. Inspect smear preparations. Report preliminary results only after acid-fast smear evaluation.
4. Follow the established procedure(s) for reporting results.

RECOMMENDED ACTIONS:

a) If AFB positive, subculture to solid media and report as: instrument-positive, AFB positive, ID pending. Subculture and identify organisms according to laboratory protocol.

b) If microorganisms other than acid-fast bacilli are present, report as: instrument-positive, AFB-negative, Contaminated. Refer to user's Manual, for tube decontamination procedure.

c) If no microorganisms are present on the smear, re-enter the tube into the instrument as an ongoing negative and allow the vial to complete testing protocol. No reportable result.

ANNEXURE – VIA

MGIT DRUG SUSCEPTIBILITY TEST

MATERIALS: MGIT 960 SIRE Kit containing one vial each lyophilized drug and SIRE Supplement vial. MGIT960 INH 0.4 Kit containing one vial each lyophilized drug and two vials of SIRE Supplement. MGIT 7 mL Mycobacteria Growth Indicator Tubes, ancillary culture media, reagents, quality control organisms and laboratory equipment as required for this procedure.

PROCEDURE

1. Label three 7 mL MGIT tubes for each test isolate. Label one as GC (Growth Control), one as INH, one as RIF. Place the tubes in the correct sequence in the appropriate size AST set carrier.

2. Aseptically add 0.8 mL of BACTECMGIT SIRE Supplement to each tube.

3. Aseptically pipette, using a micropipette, 100 µL of 8.3 µg/mL MGIT INH solution to the labeled MGIT tube.

4. Aseptically pipet 100 µL of 83 µg/mL MGIT RIF solution to the labeled MGIT tube.

5. No antibiotics should be added to the MGIT GC tube.

GROWTH CONTROL TUBE PREPARATION AND INOCULATION

1. Aseptically pipet 0.1 mL of the organism suspension into 10 mL of sterile saline to prepare the 1:100 Growth Control suspension.
2. Mix the Growth Control suspension thoroughly. Inoculate 0.5 mL of the 1:100 Growth Control suspension into the MGIT tube labeled “GC.”

ANNEXURE – VIB

DRUG-CONTAINING TUBE INOCULATION

1. Aseptically pipet 0.5 mL of the organism suspension into each of the TWO remaining drug tubes (INH and RIF).
2. Tightly recap the tubes. Mix tubes thoroughly by gentle inversion 3 to 4 times.
3. Enter the AST set into the BACTECMGIT 960 using the AST set entry feature.
4. Streak 0.1 mL of the organism suspension to a Trypticase™Soy Agar with 5% Sheep Blood (TSA II) plate. Enclose in a plastic bag. Incubate at 35 - 37°C. 9.Check the blood agar plate at 48 h for bacterial contamination.
5. Control strain M.tuberculosis ATCC™27294

Result will be interpreted and recorded according SOP.

ANNEXURE – VIIA

RSC CULTURE PROCEDURE

1. All the samples will be subjected to NALC—NaOH digestion and decontaminated process (modified Petroff's method) and centrifuge at 3000 rpm for 15 minutes. Supernatant will be discarded and sediment will be processed. **[Cruickshank R, 1997]**.

2. One smear will made at the centre of a slide (Slide-1), it was fixed and Z N staining will be done. The reading will be taken and record as smear control slide.
3. The second smear will be made at the lower 1/3rd of the slides (Slide -2) which is split longitudinally. The smear will be spread evenly, 2cm X 3cm in size and is neither too thick nor too thin.
4. The smears will be air dried dipped into the Makarthy tube which contained RSC (I & II) medium, so that the smears remained dipped in the medium. The inoculated slide will be incubated at 37 ^{o}C.
5. After incubation the slide will be taken out and will be dipped in absolute methyl alcohol to fix the smear and kill of mycobacteria which may be present on the slide surfaces.
6. The slides will be then air dry and will be later stained by ZN staining.

OBSERVATION OF SLIDE

The smear will be first focused under 4X objective and observe for any bright red or pink spot. Once the bright red or pink spot observed change the objective lens and observe the same field under 10X and 100X to confirm bacterial morphology.

ANNEXURE – VIIB

The growth will be recorded as mentioned below: The readings of the 0 grades will be taken as negative and those of the grades 1-4 will be taken as positive.

Grade 0- No division of AFB as compared to slide 1

Grade 1+ Small clumps –up to 4 bacilli were present but absent in the control

Grade 2+ large clumps of bacilli

Grade 3+ large clumps with some cord formation

Grade 4+ Micro colonies with good cord formation

[53]

The contamination of bacteria or fungus will be observed daily and recorded.

PREPARATION OF MEDIUM FOR RSC-I

Modified Middlebrook 7H9 broth base	5.9 gm
Casein peptone	1.25g
Distilled water	1000 mL

Mix the ingredients in a conical flask and distribute 7 ml in Makarthy tubes and store the tubes at 4 C.

RSC 1 MEDIUM:

1. Reconstitute a lyophilized vial of PANTA antibiotic mixture (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin) with15 mL of Growth Supplement containing 50.0 gm Bovine Albumin, 20.0 gm Dextrose, 0.03 gm Catalase, 0.1gm Oleic Acid. (Bicton and Dikson)
2. Add 0.8 mL of OADC growth supplement and PANTA antibiotic mixture prior to the inoculation.
3. Each vial of PANTA contains a lyophilized mixture of the antimicrobials with the concentrations, as follows: Polymyxin B (6,000 units), Amphotericin B (600 µg), Nalidixic Acid (2,400 µg), Trimethoprim(600 µg), Azlocillin (600 µg).

ANNEXURE – VII C

RSC 2 MEDIUM:

1. Prepare 4 % malachite green stock solution by dissolving 4 gram of malachite powder in 100 mL of deionised distilled water store in refrigerator at $8^{O}C$.
2. Add 1 mL 4 % malachite green solution in 1000 mL of Middlebrook 7H9 broth.
3. Distribute 70 ml broth in Makarthy tubes and autoclave the tubes at 15 lbs for 15minutes.
4. Before inoculation add 0.8 mL of OADC growth supplement.

ANNEXURE – VIII

SLIDE DRUG SUSCEPTIBILITY TEST

MATERIALS: Lyophilized Rifampicin and Isoniazid drugs and OADC growth supplement (Bicton and Dikson). Makarthy tubes containing 7 mL RSC media, reagents, quality control organisms and laboratory equipment as required for this procedure.

PROCEDURE

1. Label three Makathy tubes containing 7 ml of RSC medium. Label one as GC (Growth Control), one as INH, one as RIF.

2. Aseptically add 0.8 mL of growth supplement to each tube.

3. Aseptically pipette, using a micropipette, 100 µL of 8.3 µg/mL INH solution to the labeled Makathy tube.

4. Aseptically pipette 100 µL of 83 µg/mL RIF solution to the labeled Makathy tube.

5. No antibiotics should be added to the GC Makathy tube.

SLIDE PREPARATION AND INOCULATION

6. Concentrate the sputum sample by modified Petroff's method in conical tube.
7. Add 10 ml of RSC medium and incubate it for 24 hours.
8. Centrifuge the tube at 3000 rpm for 15-20 minutes and discard the supernatant.
9. Take three new glass slides label it as GC, INH and RIF.
10. Sterilize these slide in hot air oven at 180 ^{o}C for 15 minutes.
11. Pipette 10 µl of concentrated sample and make (2 x 2 mm) smear on lower 1/3rd of slides.
12. Allow the smear to air dry then place it in labeled Makarthy tube.
13. Incubate the tubes at 35-37 ^{o}C.
14. After incubation the slide will be taken out and dipped in absolute methyl alcohol to fix the smear and kill of mycobacteria which may be present on the slide surfaces.
15. The slides will be then air dry and will be later stained by ZN staining.
16. Control strain M.tuberculosis ATCC™27294. Growth if any will be recorded.

ANNEXURE – IX

GENEPROBE ASSAY

The Enhanced Amplified Mycobacterium Tuberculosis Direct Test (E-MTD. Gen-Probe, San Diego, California) (BioMerieux) combines isothermal transcription mediated amplification of a portion of 16 sRNA with a detection method that uses hybridisation probe specific for M. tuberculosis.

PROCEDURE

1. Prepare the reagents according to literature.
2. Take 50 µl specimen dilution buffer in lysing tube (1).
3. Add 50 µl amplification reagent and cover this with 200 µl paraffin oil.
4. In another lysing tube (2) take 450 µl decontaminated specimen, vortex it for 15 seconds.
5. Put the above lysing tube in sonicator for 15 minutes to break the cells.
6. Add 25 µl sonicated specimen in Tube (1).
7. Set the thermocycler and place the tube (1) in thermocycler for amplification.
8. After overnight amplification add hybridising reagent and vortex three times.
9. Incubate the tubes at 60 ^{O}C for 15 minutes for hybridization.
10. Add 300 µl selection reagent, vortex three times and incubate at 60 ^{O}C for 15 minutes.
11. Keep the tube at room temperature for 5 minutes.
12. Detection using the luminometer with the MTD protocol.

ANNEXURE – X

LINE PROBE ASSAY

The GenoType® MTBDRplus (Hain Lifescience, Nehren, Germany) LPA will be perform in BSL III laboratory.

The procedure of LPA is divided into three procedures

(1) DNA extraction from NaOH-NALC decontaminated smear positive specimens.

(2) A multiplex Polymerase Chain Reaction amplification (PCR)

(3) The reverse hybridization (including detection and evaluation).

The test will be performed according to FIND Molecular diagnosis of drug resistant tuberculosis by LPA laboratory manual. []

***ANNEXURE* – XI**

Rama Medical College Hospital & Research Centre

Mandhana Kanpur

CONSENT FORM

I .....son/daughter/wife of.....exercising my free power of choice, hereby give my consent to be included in the study "**Evaluation of A cost effective Rapid Slide Culture technique for early Diagnosis and Drug Susceptibility Testing of Tuberculosis**". I hereby give my consent for information related to me to be reported in the research study.

I understand that my name and identity will be concealed.
Once signed, I cannot revoke my consent.

(To be explain in detail in native language)

सहमति फार्म

मैं .....पुत्र /पुत्री /पत्नी/..... स्वयं इस अध्ययन में भाग ले रहा/रहीं हूं। मैं स्वयं मुझेसे संबंधी सूचना इस अध्ययन कार्य में प्रकाशित करने की सहमति देता/देती हूं। मैं समझता/ समझती हूं कि मेरा पहचान गोपनीय रखी जाएगी।

एक बार हस्ताख्रर के पश्चात मैं सहमति रद्द नहीं कर सकता/ सकती हूं।

सहभागी

नामः.....

हस्ताख्रर/अगुठे का निशानः.....

दिनाकः.....